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Antioxidant activity of winter melon (*Benincasa Hispida*) seeds using conventional soxhlet extraction technique

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Abstract: In this study, the effect of different solvent including ethanol, n-hexane and ethyl acetate on antioxidant activity and total phenolic content (TPC) of winter melon (*Benincasa hispida*) seeds extract was investigated using conventional Soxhlet extraction (CSE). DPPH and ABTS scavenging activity and TPC results indicated that the seed extracts obtained using ethanol possessed the highest antioxidant activity and followed by ethyl acetate and n-hexane. By considering obtained results, it was clear that there was a high positive correlation between TPC and antioxidant activity. Linoleic acid forms a significant percentage of unsaturated fatty acids of the seed extract (60.6%). It is well known that essential fatty acids including linoleic acid and linolenic acid which are detected in extracts play important roles in preventing many disease and abnormal differentiation problems. *B. hispida* seeds are potential source of natural antioxidant compounds to replace synthetic antioxidants.

Keywords: Winter melon (*Benincasa hispida*), conventional soxhlet extraction (CSE), antioxidant activity, essential fatty acids

Introduction

The continuous formation of free radicals in humans' body can be controlled naturally by different beneficial compounds known as antioxidants (Pietta, 2000). Oxidative stress can be caused in result of free radicals formation (Nakiboglu *et al*., 2007). Aging and different chronic diseases including diabetes, cancer and cardiovascular diseases could be caused by oxidative stress (Halliwell *et al*., 1992). Free radicals are stabilized or deactivated by antioxidants before they attack cells. Antioxidants are important factor to maintain optimal cellular and human body health. Furthermore, fortification of food formulation by adding antioxidant compounds lead to prevention of oxidative reactions which adversely affect food quality attributes. Several epidemiologic studies revealed that consumption of foods containing high amount of antioxidant compound lowering the risk of human disease occurrence (Aruoma 1998). Several synthetic antioxidants are commercially accessible. By considering potential health risks and toxicity of synthetic antioxidants like butylhydroxytoluene (BHT) and butylhydroxyanisole (BHA), there is a great demand by consumers to usage of natural antioxidants (Ito *et al*., 1986).

Benincasa hispida which known as winter melon belongs to a family of *Cucurbitaceae* and its origin can be tarced to south-east Asia where it has been cultivated for at least 2000 years. It is a very popular vegetable in China, India, the Philipines, Thiland, Vietnam and elsewhere in Asia. It is also found in areas outside Asia where people of Asian origin congrate (Grover *et al*., 2001). It was suggested that winter melon is useful for controlling of nervous disorders, ulcer healing and acid neutralizing (Sharma 1984; Warier 1994). On the other hand, the expectorant effect of the *Benincasa hispida* seeds extract due to facility of mucus secretion which prevents gastric ulcer was pointed out by Kim and Shin (1999) and Grover *et al*., (2000). In addition, *Benincasa hispida* seeds extract also could enhance immunoreactions result in histamine secretion inhibition (Yoshizumi *et al*., 1998; Lee *et al*., 2005).

Based on our knowledge, no information is available on winter melon (*Benincasa hispida*) seed extracts antioxidant activity and its fatty acid composition which obtained by using conventional Soxhlet extraction (CSE) method. Therefore, this study presents the antioxidant activity and fatty acid composition of winter melon (*Benincasa hispida*) seed extracts according to CSE method by using different solvents. Furthermore, for the first time, the effect of different solvents on the radical scavenging activity and total phenolic content (TPC) of seeds extracts were evaluated.

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Materials and Methods

Plant material and reagents

Whole winter melon (*Benincasa hispida*) fruits were obtained locally in Serdang, Selangor, Malaysia. Seeds were separated, raised using tap water and dried by means of a ventilated oven (1350FX, USA) at 40℃ for 24 h. The prepared seeds were stored in a dark place at ambient temperature. The seed powder was produced using grinder mill (MX-335, Panasonic, Malaysia).

Ethanol (EtOH, 99.5%, analytical grade), ethyl acetate ($CH_3COOCH_2CH_3$, analytical grade) and *n*-hexane (analytical grade) were provided by Scharlau Chemical, European Union. Fatty acid methyl ester (FAME) standards were obtained from Sigma–Aldrich (St Louis, MO, USA). Sodium methoxide, butylated hydroxytoluene (BHT), 2-20Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid and Folin-Ciocalteu reagent (FCR) were bought from Fisher company (Pittsburgh, PA, USA).

Conventional soxhlet extraction (CSE)

Five gram of grounded winter melon (*Benincasa hispida*) seed was put into extraction thimbles which covered with wood and then transferred into a Soxhlet apparatus (Figure 1). Different extraction solvent (150 mL) was added to each flask, which was connected to the extractor. Each extraction was performed in triplicate during 6 hr. The temperature of extraction corresponded with the boiling point of the different solvent in use. After CSE was completed, solvent was removed at 40ºC using a rotary evaporator (A-1000S, Eyela, Japan) whereas the excess solvent was eliminated by using drying procedure at 40ºC for 1 h. The extracts were kept under nitrogen (99.9%) until the analysis.

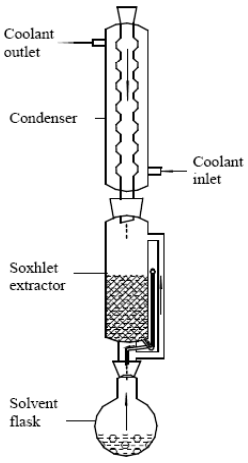


Figure 1. Experimental Soxhlet extraction apparatus (Li *et al*., 2004)

Antioxidant activity of extracts determination

Antioxidant activity of seed extracts under different conditions were assessed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Sanchez-Moreno *et al*., 1998; Turkmen *et al*., 2006; Lafka *et al*., 2007; Sanchez-Vicente *et al*., 2009) and ABTS radical scavenging assays (Gan and Latiff, 2011).

DPPH radical scavenging activity determination

This assay was carried out according to Zengin *et al*. (2010) with some modifications in which increase in absorbance due to reduction of the DPPH• radical determined spectrophotometrically at 515 nm for 60 min based on preliminary experiments. In this method, a total of 0.1 mg/ml of the extracts and synthetic antioxidants (catechin and BHT) in ethanol were added into 3 ml of ethanol solution of DPPH. The mixture was vortex for 20 s at room temperature. The blank sample (A_{blank}) absorbance which contains 0.1 ml ethanol instead of extracts was also carried out. Absorbance measurements were done immediately in a 10 mm quartz cell from 1 min up to 60 min with 10 min intervals using a UV-visible spectrophotometer (Thermo 4001/4 UV–Vis Spectrophotometer, Thermo Fisher Scientific). The percentage of inhibition was measured according to the equation presented by Gan and Latiff (2011).

$$\% \text{ inhibition} = 100 \times (A_{blank} - A_{sample}) / A_{blank}$$

ABTS radical scavenging activity determination

2, 20-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) assay was carried out according to the method of Cai *et al*. (2004). The $ABTS^{\cdot+}$ solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulphate and then incubated in the dark at room temperature for 16 h. It was diluted with 80% (v/v) ethanol to obtain an absorbance of 0.700 ± 0.005 at 734 nm. A 3.9 ml of $ABTS^{\cdot+}$ solution was mixed with 0.1 mg/ml sample vigorously. The blank sample was prepared using ethanol instead of extract and its absorbance (A_{blank}) was recorded at 734 nm for 10 min with 2 min intervals at ambient temperature. The ABTS (%) was measured using the following equation (Gan and Latiff, 2011):

$$\%ABTS_{sc} = (A_{blank} - A_{sample}) \times 100 / A_{blank}$$

TPC determination

The TPC of extract was determined using Folin–Ciocalteu reagent (FCR) according to Singleton *et al*. (1999) with some modifications. In a test tube, 1.0 mL of deionised water, 1 ml of Folin-Ciocalteu reagent (diluted 10 fold with distilled water) was added to

10 mg of extract. After 5 min at room temperature, 7.5 ml of 60 mg/ml of aqueous carbonate sodium (Na_2CO_3) solution was also added. Again, the tube was kept for 30 min. The changes of color was assessed spectrophotometerically by measuring absorbance at 765 nm (Thermo 4001/4 UV–Vis, Thermo Fisher Scientific) since maximum absorbance was obtained. The standard curve prepared at different concentrations of gallic acid (50–500 ppm) to determine the TPC of the extract as mg gallic acid equivalent and reported as mg GAE/g extract. Determination was performed trice on each sample.

Fatty acid methyl esters (FAMEs) preparation

The FAMEs profiles in the obtained extracts with different extraction conditions were analysed by using gas chromatography-mass spectrometry (GC-MS) and gas chromatography (GC). The samples were brought to the temperature of 50–60ºC and homogenized thoroughly before taking a test sample to obtain the FAME. A 100 µL of the test sample was mixed with 1 ml n-hexane in a 2 ml vial. Then, 1 µL aliquot of sodium methoxide was added to the vial which was mixed vigorously. The mixture first became clear and then turbid due to precipitation of sodium glyceroxide. After a few minutes, the clear upper layer of methyl ester was pipetted off and injected into the GC-MS and GC for analysis (Zaidul *et al*., 2007).

Analysis of gas chromatography–mass spectrometry (GC-MS)

The major compounds identification in the extracts was carried out by using gas chromatography-mass spectrometry (Hewlett-Packard, Aligent Technologies (6890N/G1540N) equipped with a flame ionization detector (FID). Capillary column DB 5 MS (5%-Phenyl) (30 m × 0.25 mm i.d.; film thickness 0.25 µm) was used with helium as carrier gas with flow rate of 6.8 ml/min. The temperature of oven was programmed in two stages as follows: first, from 50 to 180ºC (8ºC/min) and then from 180 to 200ºC (5 ºC/min). The NIST mass spectral library was used to identify extracts component by comparison of the mass spectra and their retention times.

Gas chromatography (GC) analysis

Quantitative analysis was carried out by using a Hewlett-Packard 6890 GC (Wilmington, DE) equipped with a flame ionization detector (FID) and a capillary column BPX70 (30 m × 0.25 mm × 0.25 µm, Victoria, Australia). Oven temperature was programmed to 115ºC/min, during 2 min; to 163ºC (4ºC/min); to 170ºC (1ºC/min) and then increased to 200ºC (10ºC/min) with 2 min holding time at this temperature. Helium was used as a carrier gas with flow rate of 1 ml/min. The volume of each injection was 1 µL. Standard methyl esters of fatty acids were used as authentic samples. The peak areas were obtained from the computer and the percentage of the fatty acid was calculated as the ratio of partial area to total area. The fatty acid determination was carried out in duplicate.

Statistical analysis

Statistical analysis was performed through analysis of variance (ANOVA) and Pearson's linear correlations at 95% of significance level ($P < 0.05$) using MINITAB 14.0. The results were expressed as mean values ± standard deviation (SD).

Results and Discussions

Antioxidant activity of extracts

Generally, it was found that the extracts possess high antioxidant activity indicating its ability to neutralize free radicals (Erasto *et al*., 2007). The antioxidant activity of winter melon (*Benincasa hispida*) seed's extracts was calculated according to the absorbencies reduction of DPPH and ABTS radicals at 515 and 734 nm, respectively. The DPPH scavenging activity of extracts during 60 min was shown in Figure 2. It was clear that the highest antioxidant activity is belonged to ethanolic extracts. The DPPH and ABTS scavenging activity assays in the current study revealed that the extract was potently active. The similar findings were also reported by Kirmizigul *et al*. (2007) and Aiyegoro and Okoh (2009). The scavenging activity (% Inhibition) of extracts on DPPH and ABTS radicals are shown in Table 1. Gill *et al*. (2010) measured antioxidant activity of methanolic extracts of Cucumis melo var. utilissmus seeds, which also belonged to the family of Cucurbitaceae, with DPPH assay. They found 49.3±0.64% scavenging of DPPH free radicals at 100 µg/ml extract concentration.

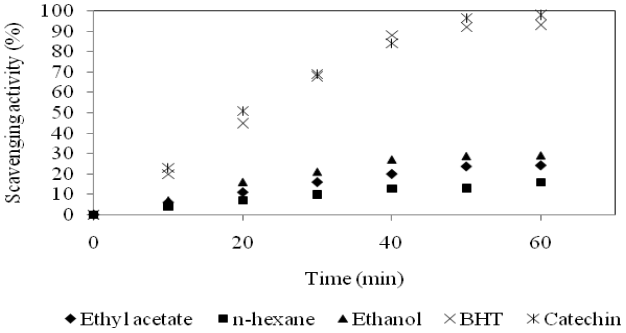


Figure 2. The effect of time on the radical scavenging activity of DPPH radicals at 0.1 mg/ml of the extract/ standard

Table 1. Free radical scavenging activity and total phenolic content (TPC) of CSE of winter melon (*Benincasa hispida*) seeds with different solvents

Extraction solvent	TPC (mg GAE/g)	$DPPH_{sc}$ (%)	$ABTS_{SC}$ (%)
n-Hexane	-	13.1[a]±0.9	12.2[a]±0.6
Ethanol (99.5%)	11.34±1.3	28.7[a]±0.7	27.0[a]±0.9
Ethyl acetate	8.23± 0.9	24.2[a]±1.3	25.4[a]±1.2
BHT[A]	-	93.43±0.6	-
Catechin	-	98.1±0.8	97.54±0.7

[A] Synthetic antioxidants
Similar superscript in each row donate insignificant difference (p>0.05).

Furthermore, the results reveal that there is no significant difference between DPPH and ABTS free radical scavenging activity while the high positive correlation between DPPH and ABTS assays (R^2=0.99) was also found. In previous study, Neo *et al*., (2010) found good correlations between ABTS and DPPH (R^2= 0.99). However, the antioxidant activity value was less than BHT and catechin (synthetic antioxidants) at the same concentrations due to thermo degradation of bioactive compounds. One of the limiting steps for applying CSE is extraction of thermo-labile compounds due to their sensitivity to high temperature (Wang and Weller, 2006). It was expected by using other extraction techniques with lower temperature and shorter extraction time these compounds protected against thermo degradation.

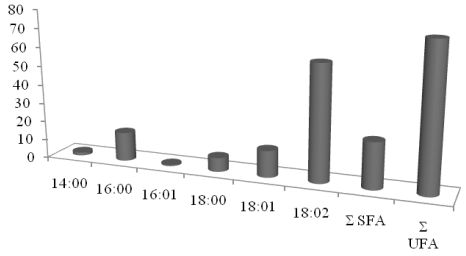
TPC determination

TPC of extracts was measured to evaluate its contribution in antioxidant activity. In TPC assays, phosphotungstic and phosphomolybdic acids react with the antioxidant compounds which demonstrate by shifting the yellow to blue color (Waterhouse, 2002; Neo *et al*., 2010). The concentrations of TPC of winter melon (*Benincasa hispida*) seed's extracts were shown in Table 1. The higher amount was obtained in the ethanol (99.5%) extracts with 11.34 ± 1.3 mg GAE/g of extract and it was reduced with using ethyl acetate 8.23 ± 0.9 mg GAE/g of extract. Amount of phenolic compounds extracted using ethanol (99.5%) suggesting higher solubility of polar compounds in ethanol. In current study by using n-hexane as solvent extraction no total phenolic content (TPC) was detected due to its lower polarity compare to ethanol. As phenols are polar compounds, it was pointed out that adding different ratio of polar solvents such as methanol and ethanol are useful to increase the polarity of extraction solvent (Heim *et al*., 2002; Ramirez *et al*., 2005). Dutra *et al*. (2008) conducted another investigation on quantification of phenolic constituents of *Pterodon emarginatus* seeds which found that it was not possible to detect the phenolic constituents in hexane extractions of seeds.

Considering to available literature, there are several studies which reported a highly positive relation between TPC and antioxidant activity in many plants and fruits (Tabart *et al*., 2009; Sim *et al*., 2010). However, the great antioxidant activity of phenolic compounds compared with vitamins C and E is reported (Guo *et al*., 2003; Sim *et al*., 2010). From the data in Table 1, it is clear that the ethanol extracts show higher DPPH and ABTS radical scavenging activity due to their higher TPC amount compare with ethyl acetate and n-hexane extract. In addition, a high Pearson's correlation coefficient between TPC and DPPH radical scavenging activity was also found (R^2= 0.99). Similar findings were also published by Duh *et al*. (1999) and Gulcin *et al*. (2004).

Fatty acid profile analysis

Figure 3 shows the fatty acid composition of winter melon (*Benincasa hispida*) seed's extract using Ethanol (99.5%). In addition to high amounts of fatty acids, it is apparent that linoleic (18:2 n-6) and oleic (18:1 n-9) are the principal fatty acid components in the seed's extracts. In fact, winter melon (*Benincasa hispida*) seeds extract mainly include unsaturated fatty acids with a clear predominance of linoleic acid. Detection of the one essential fatty acids (EFA), which should supply from dietary sources due to inability of human body for their manufacturing, reflects its significance as a good supplement in human diet (Deferne and Pate, 1996; Erasto *et al*., 2007).



	14:00	16:00	16:01	18:00	18:01	18:02	Σ SFA	Σ UFA
Fatty acids	1.6	15.3	0.68	7.4	14.1	60.6	24.3	75.38

Figure 3. Fatty acid compositions (% of total fatty acids) of winter melon (*Benincasa hispida*) seeds extract

The literature fatty acid composition of *Citrullus lanatus* and *Cucumis melo* var. *flexuosus* (which belongs to family of *Cucurbitacea*) was presented in Table 2 for comparison with winter melon seed extracts fatty acid composition (Nyam *et al*., 2009; Mariod *et al*., 2009). It is well known that EFAs including linoleic acid α- inolenic acid play important roles in preventing many disease and abnormal differentiation problems (Kirmizigul *et al*., 2007).

Table 2. Fatty acid compositions (% of total fatty acids) of winter melon (*Benincasa hispida*) seed extracts compared with other seeds

Fatty acid	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	ΣSFA	ΣUFA
Benincasa hispida	1.6	15.3	0.68	7.4	14.1	60.6	-	-	24.3	75.38
Citrullus lanatus	-	12.4	-	7.5	17.1	63.1	1.1	0.3	19.2	80.8
Cucumis melo var. flexuosus	0.1	12.9	-	6.0	19.4	61.4	-	-	19.9	81.6

Linoleic acid (an omega-6 fatty acid) as a metabolic precursor of eicosanoids which forms important lipids like prostaglandins play an important role in inflammation, immunity and blood clotting (Bourre *et al*., 1993). α-Linolenic acid (anomega-3 fatty acid) as a precursor of the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been reported to play an important role in infants brain development (Makrides *et al*., 1995; Erasto *et al*., 2007). Generally, it has been found that the polyunsaturated fatty acids have positive effect in the reducing of coronary heart disease (Oliver, 1997). Furthermore, a positive correlation between EFA and antioxidant activity was found by Ismail *et al*., (2004) and Kirmizigul *et al*., (2007).

Conclusion

In the current study, for the first time, the CSE technique using different solvents including n-hexane, ethyl acetate and ethanol (99.5%) were applied to extract valuable compounds from winter melon (*Benincasa hispida*) seeds and to evaluate the influence of solvents on antioxidant activity and TPC of the extracts. It was found that higher amount antioxidant activity was obtained in the ethanol (99.5%) extracts whereas a high positive correlation between TPC and antioxidant activity was also obtained. The fatty acid composition of extract obtained using Ethanol (99.5%) was analysed to determine its major fatty acids. The winter melon seed extract was contained reasonably high unsaturation fatty acids (75%) which make it promising option for commercial utilization. It was found that the main constituents of CSE extracts were linoleic acid (LA) and oleic acid. Also, α-linolenic acid (ALA) was identified in obtained in Ethanol (99.5%) extracts. Therefore, the presence of essential fatty acids in winter melon's seed demonstrates its importance in the human diet.

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